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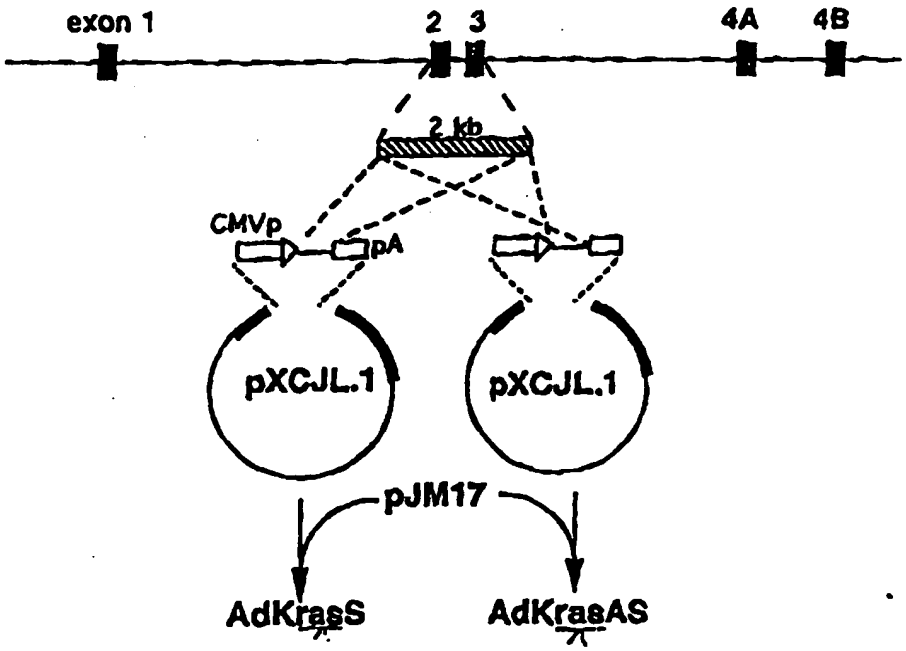

(57) Abstract

A variety of genetic constructs are disclosed that will find both *in vitro* and *in vivo* use in the area of tumor biology and cancer therapy. In particular, adenoviral expression vectors are provided that contain a K-*ras* nucleic acid positioned antisense to regulatory control regions. In one embodiment, the adenoviral expression vector is a replication-deficient adenoviral vector lacking the E1 region and containing a K-*ras* nucleic acid. Also provided are methods for the inhibition of cancer cell proliferation.

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DESCRIPTION

ADENOVIRUS-ANTISENSE K-*ras* EXPRESSION VECTORS AND THEIR APPLICATION IN CANCER THERAPY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of tumor biology. In particular, the invention relates to a polynucleotide encoding an antisense construct that targets a known oncogene. In one embodiment, the invention relates to adenovirus expression vectors encoding an antisense K-*ras* and their use in inhibiting cancer.

2. Description of the Related Art

Cancer is one of the leading causes of human disease, being responsible for 526,000 deaths in the United States each year. Lung cancer alone kills more than 140,000 people annually in the United States. Recently, age-adjusted mortality from lung cancer has surpassed that from breast cancer in women. Although implementation of smoking-reduction programs has decreased the prevalence of smoking, lung cancer mortality rates will remain high well into the twenty-first century. Unfortunately, current treatment methods for cancer, including radiation therapy, surgery and chemotherapy, are known to have limited effectiveness. The rational development of new therapies for lung cancer largely will depend on gaining an improved understanding of the biology of cancer at the molecular level.

With advances in molecular genetics and biology, it has become evident that altered expression of normal genes can lead to the initiation of transforming events that result in the creation of cancer cells. The conventional therapy for malignancy, such as chemotherapy and radiation, has focused on mass cell killing without specific targeting, often resulting in damaging side effects. A new direction in cancer therapy is to deliver a normal gene to replace or correct the mutated gene, thereby altering the malignant phenotype of transformed cells. Several expression vectors have been developed in order to deliver a gene into somatic cells with high efficiency.

The *ras* gene family is, perhaps, the best characterized of the oncogene families. Most of the identified transforming genes in human carcinomas have been members of the *ras* family, which encode immunologically related proteins that have a molecular weight of 21,000 (designated p21). Nucleotide sequence analysis of several *ras* mutants reveals different mutations, but amino acid residues 12 and 61 appear to play an important role. Tabin (1982); Der and Cooper (1983); Yuasa *et al.* (1983).

Mutations of the *ras* gene family are found in more than 30% of human carcinomas, especially those of pancreas, colon and lung. The role of these mutations in tumorigenesis, invasion and metastasis has been well documented. Barbacid (1985); Bos (1989). Protein products of mutated *ras* genes permanently transduce a strong mitogenic signal to stimulate cell proliferation. Therefore, blocking mutated *ras* has a clear antitumor potential, and different strategies have been used to achieve this objective. For example, the neoplastic phenotype associated with mutated *ras* genes has been reversed by antibodies to p21, by fragments of natural

p21 ligands (*e.g.*, NF1 and c-Raf-1), and by dominant negative *ras* mutants. Mulcahy *et al.* (1985); Fridman *et al.* (1994); Ogiso *et al.* (1994). At the mRNA level, strategies have been based on anti-H-*ras* ribozymes and antisense oligonucleotides. Kashani-Sabet *et al.* (1992); Brown *et al.* (1989); Debus *et al.* (1990). In addition, targeting of DNA has been accomplished by homologous recombination. Shirasawa *et al.* (1993).

Up to 30% of all lung adenocarcinomas have a mutated *ras* gene, and more than 90% of these mutations occur in the K-*ras* gene. In the neoplastic development of lung tumors, K-*ras* mutations may arise prior to invasion and can easily be detected in sputum samples. The presence of this mutation correlates with a poor clinical outcome. Initial studies have shown that K-*ras* expression in tumor cell lines can be inhibited by transfection of a plasmid construct that expresses a K-*ras* antisense RNA. This K-*ras* construct was then inserted into a retroviral vector and similar results were achieved following infection of tumor cells and in an orthotopic nude mouse model. Mukhopadhyay *et al.* (1991); Georges *et al.* (1993). Despite these results, the retroviral system is not without its limitations. For example, vector-borne genotoxicity is associated with integration. Retroviruses also are unstable, require specific receptors for entry in to cells and replicate only in actively proliferating cells. Thus, there remains a need for improved gene therapeutic compositions for use in anticancer treatments.

3. Summary of the Invention

The present invention addresses the need for improved therapy for lung and other K-*ras*-associated cancers by providing adenoviral expression vectors containing a polynucleotide encoding a K-*ras* antisense

transcript. It also is an object of the present invention to provide methods for the use of such compositions and, in particular, use in the treatment of cancer.

The present invention encompasses adenovirus expression vectors that comprise a promoter functional in eukaryotic cells and a polynucleotide encoding a K-*ras* antisense construct, the polynucleotide being under transcriptional control of the promoter and positioned such that the transcript produced is antisense. In a preferred embodiment, the adenovirus lacks at least a portion of the E1 region. In another embodiment, the adenoviral expression vectors further comprise a polyadenylation signal. In yet another embodiment, the constructs further comprise a selectable marker.

In certain embodiments, the polynucleotide is derived from the genome. In other embodiments, the polynucleotide is a cDNA or synthetically generated polynucleotide. Still other embodiments include a combination of cDNA and genomic DNA, for example, in a mini-gene construct. Further embodiments include fragments of K-*ras* that correspond to introns and/or splice junctions.

The present invention also includes pharmaceutical compositions comprising an expression vector with a promoter functional in eukaryotic cells and a polynucleotide encoding a K-*ras* antisense transcript, along with a pharmaceutically acceptable buffer, solvent or diluent. In certain embodiments, the expression vector and pharmaceutically acceptable buffer, solvent or diluent are supplied in a kit.

The present invention further comprises a method for inhibiting K-*ras* function in a cell. This method

comprises contacting such a cell with an expression vector as described above, wherein the polynucleotide is positioned in an antisense orientation with respect to the promoter. In an exemplary embodiment of the invention, the cell is a transformed cell and the contacting reverses the transformed phenotype. In a further embodiment, the cell is a lung, pancreas or colon cancer cell.

Another embodiment of the invention is a method of treating a mammal with cancer. This method comprises administering to an animal a pharmaceutical composition comprising an expression vector having a promoter functional in eukaryotic cells and a polynucleotide encoding a K-*ras* antisense transcript, in a pharmaceutically acceptable buffer, solvent or diluent. In a particular embodiment of the invention, the mammal is a human. In another embodiment, administering is via intratumoral instillation. In a further embodiment, the cancer is lung cancer.

4. Brief Description of the Drawings

FIG. 1 - Adenoviral Vector Construction. A 2 kB genomic fragment containing exons 2 and 3 and intron 2 of the K-*ras* protooncogene was cloned between the CMV promoter and the SV40 polyadenylation signal in sense and antisense orientations. These expression constructs were inserted into the polylinker site of pXCJL.1, which contains the left arm of Adenovirus type 5 (Ad5) with the exception of an E1 deletion. These plasmids were individually cotransfected into 293 cells with pJM17, a non-packageable Ad5 genome, which results in rescue of the constructs into the Ad5 genome and renders the recombinant packageable.

FIG. 2 - Growth Curve of Transduced H460a Cell *In Vitro*. At the indicated days following initial infection (MOI of 100 pfu/cell, day 0), cells were incubated with [^{3}H]thymidine for 4 h and harvested, and the incorporated radioactivity was counted (cpm). The plot represents combined data from three studies. Similar curves were obtained by cell counting ($P<.001$) by analysis of variance test.

5. Detailed Description of the Preferred Embodiments

Previous studies have shown that regions of the K-*ras* gene can be used as antisense constructs to inhibit the expression of the K-*ras* product and, in so doing, reverse the transformed phenotype of tumor cells in which the K-*ras* product is aberrant, either in level of expression or in sequence. This has been accomplished by using both expression plasmids *in vitro* and retroviral vectors *in vivo*. A particular region of the K-*ras* gene was used, spanning exons 2 and 3 and including the intervening intron 2, which allowed discrimination between the oncogene and other *ras*-related sequences.

Here, those studies are extended to the use of a genetically engineered adenovirus expression vector. An adenoviral vector carrying a 2 kB fragment of the K-*ras* protooncogene, inserted in an antisense orientation to the construct promoter, was used to infect H460a lung cancer cells. Efficient transfer and high level expression from the construct were observed. At a multiplicity of infection of 100, 65% of cells were transduced and K-*ras* production was reduced by 70%. This resulted in a 40% inhibition of monolayer growth and, interestingly, a 90% inhibition of colony formation.

Thus, the present invention involves the use of adenoviral expression vectors in the reversal of the

transformed state of certain tumor cells. The adenovirus genome provides an advantageous framework in which to insert a therapeutic gene, in this instance, an antisense polynucleotide for a K-*ras* antisense construct. Preferred forms of the virus are replication defective and can only be grown on special, helper cell lines that provide the missing replicative functions in *trans*. Such an engineered adenovirus can be propagated *in vitro* to high titers for use in treating cancer cells.

It is proposed that antisense constructs containing introns bind to "sense" intron regions found on the RNA transcript of the gene, and affect proper RNA processing. Thus, subsequent translation of protein-coding RNA's into their corresponding proteins is inhibited or prevented. The use of antisense introns may prove advantageous, in certain situations, because genetic diversity in non-coding regions may be higher than in coding regions.

As used herein, the term "intron" is intended to refer to gene regions that are transcribed into RNA molecules, but processed out of the RNA before it is translated into a protein. In contrast, "exon" regions are those which are translated into protein.

Thus, where one seeks to selectively inhibit a particular gene or genes over a related gene, as is the case with *ras* genes, one embodiment proposes to target distinct intron regions. A "distinct" intron region, as used herein, is intended to refer to an intron region that is sufficiently different from an intron region of another gene such that cross hybridization would not occur under physiologic conditions. The intracellular concentration of monovalent cation is approximately 160 mM (10 mM Na^+; 150 mM K^+). The intracellular concentration of divalent cation is approximately 20 mM (18 mM Mg^+; 2 mM Ca^{++}). The intracellular protein

concentration, which would serve to decrease the volume of hybridization and, therefore, increase the effective concentration of nucleic acid species, is 150 mg/ml. Constructs can be tested *in vitro* under conditions that mimic these *in vivo* conditions. Typically, where one intron exhibits sequence homology of no more than 20% with respect to a second intron, one would not expect hybridization to occur between antisense and sense introns under physiologic conditions.

The following description defines the invention in detail.

A. K-*ras* AND K-*ras*-RELATED ANTISENSE POLYNUCLEOTIDES

The term "K-*ras* antisense polynucleotide" is intended to refer to molecules complementary to the RNA of K-*ras* or the DNA corresponding thereto. "Complementary" polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing,

transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of a intron/exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is includes simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As used herein, the terms "complementary" or "antisense sequences" mean polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides according to the present invention may encode an entire K-*ras* gene or a portion of K-*ras* that is sufficient to effect antisense inhibition of *ras* expression. The polynucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

The DNA and protein sequences for K-*ras* are provided below. It is contemplated that natural variants of K-*ras* exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided polynucleotide sequence for K-*ras* but, rather, includes use of any naturally-occurring

variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, *i.e.*, avoid unwanted antisense inhibition of K-*ras*-related transcripts. The present invention also encompasses chemically synthesized mutants of these sequences.

Another kind of sequence variant results from codon variation. Because there are several codons for most of the 20 normal amino acids, many different DNA's can encode the K-*ras* shown in FIG. 4. Reference to the following table will allow such variants to be identified.

TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

Allowing for the degeneracy of the genetic code, sequences that have between about 50% and about 75%, or between about 76% and about 99% of nucleotides that are identical to the nucleotides disclosed herein will be preferred. Sequences that are within the scope of "a K-*ras* antisense polynucleotide" are those that are capable of base-pairing with a polynucleotide segment

containing the complement of the K-*ras* sequences disclosed herein as SEQ ID NO:1 through SEQ ID NO:7 under intracellular conditions.

As stated above, although the K-*ras* antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of base-pairing. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs will be used.

While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense polynucleotide is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether the endogenous

gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in K-*ras* DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense polynucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense polynucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone.

The nucleotide and amino acid sequences of K-*ras* are as follows:

The following sequence includes a genomic fragment of K-*ras* from base 67 to base 1961. This genomic fragment includes exon 2. The exon begins at base 618 and ends at base 796. The underlined sequences are examples of oligonucleotide primer hybridization sequences to be used in the practice of the present invention.

ATGCAGAGGC CGAGGCCGCC TCGGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT
TTGGAGGTAT GAACAGACAC TTAAGTTATT TCCACATTTG GGGTATTATA AATAGTGCTG
CTGCGAACAT TGGTGTACAT GTATCTGTTT GAGTCCCTGT NNTTAGTTAT TTTGGTTATA
TACCTAGGAA TGGAATNGCT GATCATATGG TAATTCTGTG TTTAACTTTN TGAGGAACTA
CCACTGTTTT CCACAATGGC ATCACCCATT TTACATTCCC ACCAGCAATG CACAAAGATT
TCAGTGTCTG TATCCTTGCT AACACTTATT TTCCATTTTN TGAGTTTTNA TGTTTTGGTC
TNNTTAATAA TAGCCAATCC TAATGGGTAT GTGGTAGCAT CTCATGGCTT GGATTTTATT
TTCCTGACTA TTGATGATGT TGAGCATCTT TTCAGGTGCT TAGTGGCCAT TTGTCCGTCA
TCTTTGGAGC AGGAACAATG TCTTCAAGTC CTTTGCCCAT TTTTAAATTG AATTTTTTGT
TGTTGAGTTG TATATAACAC CTTTTTTGAA GTAAAAGGTG CACTGTAATA ATCCAGACTG
TGTTTCTCCC TTCTCAGGAT TCCTACAGGA AGCAAGTAGT AATTGATGGA GAAACCTGTC
TCTTGGATAT TCTCGACACA GCAGGTCAAG AGGAGTACAG TGCAATGAGG GACCAGTACA
TGAGGACTGG GGAGGGCTTT CTTTGTGTAT TTGCCATAAA TAATACTAAA TCATTTGAAG
ATATTCACCA TTATAGGTGG GTTTAAATTG AATATAATAA GCTGACATTA AGGAGTAATT
ATAGTTTTTA TTTTTTGAGT CTTTGCTAAT GCCATGCATA TAATATTTAA TAAAAATTTT
TAAATAATGT TTATGAGGTA GGTAATATCC CTGTTTTATA AATGAAGTTC TTGGGGGATT
AGAGCAGTGG AGTAACTTGC TCCAGACTGC ATCGGTAGTG GTGGTGCTGG GATTGAAACC
TAGGCCTGTT TGACTCCACA GCCTTCTGTA CTCTTGACTA TTCTACAAAA GCAAGACTTT
AAACTTTTTA GATACATCAT TAAAAAAGAA AACCATAAAA AAGAATATGA AAAGATGATN
TGAGATGGTG TCACTNTAAC AGTCTTANAN ACAATCGNGT GTATAGCATA GAATGCTGGA
TTGGATAAAC CAGTGGCATT AAAAAATTTT AAAGAATAAA AGTTTTGAAA GATTGAAGAA
GTTGGGCATT ACAGTTCTCT TAAATCTGAC CAAGCTGCAT AAAACCTATT AAAATAATCA
TTATTATGCT ATTTTATATT CTATTTCTTT GAGGGTTTAG TTTTCCCCCA AACTACATAT

TAAGCAAATG AATCACTCAG TGGCTATGTC ATATAATAAC GAGTTAGCCT AGTTATAAGA
AGTTTAACAT TTTATTTAAG AACATTGTTA CAGCATGTTT ACTGTATAGT CTAGTAATAG
AGGAAAAGAC ATTTGGGTGG GTGGTAGTGG TAGTATTTTT ATAGAGGAGT TACCAAATTT
CAGCTCTATT ATCCAAGTTT ACCCAGCTAA TGGTGTTCGG AACCGGGAAT TTGAGCCAAT
TGTGACTCTG TTGGCTGCTC TGCTCCTTCT TTTGTGCTGT GTCTTTGAAA AGTCACCTAA
AATTGTGAGG GAATGTAATT TCACCCCAAA TTTAGAGTTT ATGCACTTGT TATATTGAAA
ATGATTAACA TGTAGAAGGG CTTTTAATGG AATAAGTGGT GTAGTAACTT CAGTGTTGCC
TACCTAGAAA GGAAAATCTT TCTAGTTGTC CACTTTGTTT TTTGAAAAAG TAATATGAAA
ATTATGTTAA TGCTTTAATT CAGGTTTTTG TAAAATATTT TTTATCTTTA CACATTTAAC
ATACGTTTCT AAAATTATAG TCTGTTATAT AGCACTTTGG GTAGATCCAG CTTGGGCTGC
AGGTCGACTC TAGCTGCGAG AATAGCCGGG CGCGCTGTGA GCCGAAGTCG CCCCCGCCCT
GGCCACTTCC GGCGCGCCGA GTCCTTAGGC CGCTAGGGGG CGCCGGCGCG CGCACGATTG
GGGATAAAGG AAGCCGGGCC GGCGCGTTAT TACCATAAAA GGCAAACACT GGTCGGAGGC
GTCCCCGCGG CGCGCGGCAG GAAGCCAGGC CCCAACCCCC TCCCAACCGG GCGCCAGCCC
CGCCTCCGCC CGRTTCAAAC AGCGCCGGGT CGCGCGCGCG CACGCAGCGG CCACACCCTC
GGGCGGCCRG CGGCTCGGGC AGGAAGTGCG CAAGCGCCCG GGAGCCGCGG CGACCCCACC
CCTTCCGACC GAGCCCGCCT TCGCCCCRGC CCRGGCCGCG GCACCCCGGG CCCCAGAACG
CAGCCGCAAT TAGCGCCTTG AGTCCCRGGC CGCAGCCGCA ATTAGCGCCA ATTCCCRGCG
CGCACGCAGT TAGCGCCCAA AGGACCAGCG CGCACGCGCA TGGCGCCCCA GCCCCCACCG
GGCCTGGCGG GGGCTACGCC GCGCCCACCC TGCGATCCCC ATTGGCAAGA GCCCGGCTCA
GACAAAGACC CCGCCGGTTG CCCCCGCCCC GAGAGCGGCA CCCCCGGAGC GCGCCTCCCG
AGCGCGGCCT CGCGCCTCCG AACTGGCGTG GGGTGTCCCC CATCTCCGGA GGCCCAGGGG
CTTCTCCCGC GCCCCCCACG GCGGTCCGGT TCCCCCCCCA TGCGCCCCCC GCTGCGGCCC

AGACGGCGGC TCTGCACGGG CGAAGGGGCC GCGGCCGCAT GCCCCGGTCG GCTGGCCGGG
CTTACCTGGC GGCGGGTGTG GACGGGCGGC GGATCGGCAA AGGCGAGGCT CTGTGCTCGC
GGGGCGGACG CGGTCTCGGC GGTGGTGGCG CGTCGCGCCG CTGGGTTTTA TAGGGCGCCG
CCGCGGCCGC TCGAGCCATA AAAGGCAACT TTCGGAACGG CGCACGCGAT TGGCCCCGCG
CCGCTCACTC ACCGGCTTCG CCGACAGTGC AGCATTTTTT TACCCCCTCT CCCCTCCTTT
TGCGAAAAAA AAAAAGAGCG AGAGCGAGNA TTGAGGAAGA GGAGGAGGGA GAGTTTTGGC
GTTGGCCGCC TTGGGGTGCT GGGCCCGGGG GCTGGGGGCG CGCGCCGTGG CCCCCGCGCC
CCACGCTGGG CAGTGCCCGG TTCGGCCCCG CATGGCCAGG CCTGCCCCCG GCCTGCCCGT, SEQ ID NO:1.

The following sequences are genomic sequences from GenBank accession numbers L00045, K000652 and K000653.

The first sequence includes exon 1. The coding sequence is from base 96 to base 207, which is the exon/intron junction (in bold).

gtactggtgg agtatttgat agtgtattaa ccttatgtgt gacatgttct aatatagtca
cattttcatt atttttatta taaggcctgc tgaaaatgac **tgaatataaa cttgtggtag**
ttggagctgg tggcgtaggc aagagtgcct tgacgataca gctaattcag aatcattttg
tggacgaata tgatccaaca atagaggtaa atcttgtttt aatatgcata ttactggtgc
aggaccattc tttgatacag ataaaggttt ctctgaccat tttcatgagt, SEQ ID NO:2.

The following sequence contains exon 2 from bases 358 to 536 (in bold).

atcaccattt tacattccca ccagcaatgc acaaagattt cagtgtctgt atccttgcta
acacttattt tccatttttt gagtttttt gttttgtttt tttaataata gccaatccta
atgggtatgt ggtagcatct catggttttg attttatttt cctgactatt gatgatgttg
agcatctttt caggtgctta gtggccattt gtccgtcatc tttggagcag gaacaatgtc
ttttcaagtc ctttgcccat ttttaaattg aatttttgt tgttgagttg tatataacac
ctttttgaa gtaaaaggtg cactgtaata atccagactg tgtttctccc ttctcaggat
tcctacagga agcaagtagt aattgatgga gaaacctgtc tcttggatat tctcgacaca
gcaggtcaag aggagtacag tgcaatgagg gaccagtaca tgaggactgg ggagggcttt
ctttgtgtat ttgccataaa taatactaaa tcatttgaag atattcacca ttataggtgg
gtttaaattg aatataataa gctgacatta aggagtaatt atagttttta ttttttgagt
ctttgctaat gccatgcata taatatttaa taaaaatttt taaataatgt ttatgaggta
ggtaatatcc ctgttttata aatgaagttc ttgggggatt agagcagtgg agtaacttgc
tccagactgc atcggtagtg gtggtgctgg gattgaaacc taggcctgtt tgactccaca
gccttctgt, SEQ ID NO:3.

The following sequence contains exon 3, from base 299 to base 458 (in bold).

tctagaattt ttcagtagtt tctgttttac tattatgatc tacctgcata ttaacctatt
aggttatagt tttactatac ttctaggtat ttgatctttt gagagagata caaggtttct
gtttaaaaag gtaaagaaac aaaataacta gtagaagaag gaaggaaaat ttggtgtagt
ggaaactagg aattacattg ttttctttca gccaaatttt atgacaaaag ttgtggacag
gttttgaaag atatttgtgt tactaatgac tgtgctataa ctttttttc tttcccagag
aacaaattaa aagagttaag gactctgaag atgtacctat ggtcctagta ggaaataaat

gtgatttgcc ttctagaaca gtagacacaa aacaggctca ggacttagca agaagttatg
gaattcattt tattgaaaca tcagcaaaga caagacaggt aagtaacact gaaataaata
cagatctgtt ttctgcaaaa tcataactgt tatgtcattt aatatatcag ttttttctc
aattatgcta tactaggaaa taaaacaata tttagtaaat gtttttgtct cttgagaggg
cattgcttct taatc, SEQ ID NO:4.

The following sequence contains exon 4 from base 600 to base 723 (in bold).

acagaagacc cagtctcagc ttcacttgta taccctggaa atagactgaa aggtgttaaa
atttaaaata aaactcaagg ttccagtttc ttgactcacc tttgagattc ttttatgttt
ttgttgtttt ttaacaaagg tttcacgtcc atattttacc atttttcttc tcattctccc
ctggaggagg gtgtgggaat cgatagtata taaatcactt ttttcctaag tcaaagaagt
aatttaaagc taacttcagt ttaggcttta attccaggac tagcaaacta aaatggttgc
attaattgac aaacagatgc taatacctgt gtttaggctt gtcataatct ctcctaattc
ctaatttaaa aattttaaaa tttaattcca ttagaaaaca aaactgactt ttaagaacaa
accaggattc tagcccatat tttaaaactg catcctcagt tttattcaaa cagtctgatg
tctgtttaaa aaaaaaaaaa tctcaagctc ataatctcaa acttcttgca catggctttc
ccagtaaatt actcttacca atgcaacaga ctttaaagaa gttgtgtttt acaatgcaga
gagtggagga tgctttttat acattggtga gagagatccg acaatacaga ttgaaaaaaa
tcagcaaaga agaaaagact cctggctgtg tgaaaattaa aaaatgcatt ataatgtaat
ctggtaagtt taagttcagc acattaattt tggcagaaag cagatgtctt ttaaaggtaa
caaggtggca accactttag aactacttag gtgtagtatt ctaacttgaa gtattaaaag
ataagaaact tgtttccata attagt, SEQ ID NO:5.

The following sequence contains exon 5 from base 159 to base 275 (in bold).

gaattctaaa agtcctaata tatgtaatat atattcagtt gcctgaagag aaacataaag
aatccttttct taatattttt tccattaatg aaatttgtta cctgtacaca tgaagccatc
gtatatattc acattttaat acttttttatg tatttcaggg **tgttgatgat gccttctata**
cattagttcg agaaattcga aaacataaag aaaagatgag caaagatggt aaaaagaaga
aaaagaagtc aaagacaaag tgtgtaatta tgtaaataca atttgtactt ttttcttaag
gcatactagt acaagtggta atttttgtac attacactaa attattagca tttgttttag
cattacctaa ttttttttcct gctccatgca gactgttagc ttttaccttа aatgcttatt
ttaaaatgac agtggaagtt ttttttttcct cgaagtgcca gtattcccag agttttggtt
tttgaactag caatgcctgt gaaaaagaaa ctgaatacct aagatttctg tcttggggtt
tttggtgcat gcagttgatt acttcttatt tttcttacca agtgtgaatg ttggtgtgaa
acaaattaat gaagctt, SEQ ID NO:6.

The following sequence is a cDNA sequence of K-*ras* and the derived amino acid sequence.

```
atg act gaa tat aaa ctt gtg gta gtt gga gct ggt ggc gta ggc aag agt
 M   T   E   Y   K   L   V   V   V   G   A   G   G   V   G   K   S
gcc ttg acg ata cag cta att cag aat cat ttt gtg gac gaa tat gat cca
 A   L   T   I   Q   L   I   Q   N   H   F   V   D   E   Y   D   P
aca ata gag gat tcc tac agg aag caa gta gta att gat gga gaa acc tgt
 T   I   E   D   S   Y   R   K   Q   V   V   I   D   G   E   T   C
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ctc ttg gat att ctc gac aca gca ggt caa gag gag tac agt gca atg agg
 L   L   D   I   L   D   T   A   G   Q   E   E   Y   S   A   M   R
gac cag tac atg agg act ggg gag ggc ttt ctt tgt gta ttt gcc ata aat
 D   Q   Y   M   R   T   G   E   G   F   L   C V   F   A   I   N
aat act aaa tca ttt gaa gat att cac cat tat aga gaa caa att aaa aga
 N   T   K   S   F   E   D   I   H   H   Y   R   E   Q   I   K   R
gtt aag gac tct gaa gat gta cct atg gtc cta gta gga aat aaa tgt gat
 V   K   D   S   E   D   V   P   M   V   L   V   G   N   K   C   D
ttg cct tct aga aca gta gac aca aaa cag gct cag gac tta gca aga agt
 L   P   S   R   T   V   D   T   K   Q   A   Q   D   L   A   R   S
tat gga att cct ttt att gaa aca tca gca aag aca aga cag aga gtg gag
 Y   G   I   P   F   I   E   T   S   A   K   T   R   Q   R   V   E
gat gct ttt tat aca ttg gtg aga gag atc cga caa tac aga ttg aaa aaa
 D   A   F   Y   T   L   V   R   E   I   R   Q   Y   R   L   K   K
atc agc aaa gaa gaa aag act cct ggc tgt gtg aaa att aaa aaa tgc att
 I   S   K   E   E   K   T   P   G   C   V   K   I   K   K   C   I
ata atg taa tct g, SEQ ID NO:7.
 I   M   *, SEQ ID NO:8.
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B. ADENOVIRAL EXPRESSION VECTORS

Throughout this application, the term "adenoviral expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (i) support packaging of the construct and (ii) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in the human.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. Both ends of the viral genome contain 100-200 base pair (bp) inverted terminal repeats (ITR), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA

replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNA's for translation.

In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure. Use of the YAC system is an alternative approach for the production of recombinant adenovirus.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham, *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or

both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury, *et al.*, 1987), providing capacity for about 2 extra kB of DNA. Combined with the approximately 5.5 kB of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kB, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1 deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available adenovirus vectors at high multiplicities of infection (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagation of adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 L siliconized spinner-flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to

the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1-4 h. The medium is then replaced with 50 ml of fresh medium and shaking started. For virus production, cells are allowed to grow to about 80% confluence after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding K-*ras* at the position from which the E1 coding sequences have been removed. However, the position of insertion of the K-*ras* construct within the adenovirus sequences is not critical to the present invention. The polynucleotide encoding a K-*ras* antisense transcription unit also may be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson

et. al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

The polynucleotide encoding the K-*ras* polynucleotide typically is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced synthetic machinery, that is required to initiate the

specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved

relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a K-*ras* polynucleotide is not believed to be critical, so long as it is capable of expressing the polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the K-*ras* polynucleotide. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of polynucleotides is contemplated as well, provided that the levels of expression are sufficient to produce a growth inhibitory effect.

By employing a promoter with well-known properties, the level and pattern of expression of a polynucleotide following transfection can be optimized. For example, selection of a promoter which is active in specific cells, such as tyrosinase (melanoma), alpha-fetoprotein and albumin (liver tumors), CC10 (lung tumor) and prostate-specific antigen (prostate tumor) will permit tissue-specific expression of K-*ras* antisense

polynucleotides. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the antisense construct. For example, with the polynucleotide under the control of the human PAI-1 promoter, expression is inducible by tumor necrosis factor. Tables 2 and 3 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of K-*ras* antisense constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of K-*ras* antisense expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in the K-*ras* antisense polynucleotide expression vector (Table 2 and Table 3). Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a K-*ras* construct. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacteriophage promoters if the appropriate bacteriophage polymerase is provided, either as part of the delivery complex or as an additional genetic expression vector.

TABLE 2

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5^k_α
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
β-Globin
c-fos
c-HA-*ras*
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)

TABLE 2 (continued)

ENHANCER
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 3

Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

In certain embodiments of the invention, the delivery of an expression vector in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression vector. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion

of a drug selection marker aids in cloning and in the selection of transformants. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed along with the polynucleotide encoding K-*ras* antisense. Further examples of selectable markers are well known to one of skill in the art.

One will typically include a polyadenylation signal to effect proper polyadenylation of the antisense transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the SV40 polyadenylation signal in that it was convenient and known to function well in the target cells employed. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

C. METHODS FOR GENE TRANSFER

In order to effect expression of antisense K-*ras* constructs, the expression vector must be delivered into a cell. As described above, the preferred mechanism for delivery is via viral infection where the expression vector is encapsidated in an infectious adenovirus particle.

Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include

calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), polycations (Boussif *et al.*, 1995) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In one embodiment of the invention, the adenoviral expression vector may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an antisense K-*ras* construct may also be transferred in a similar manner *in vivo*.

Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been

developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a K-*ras* antisense construct may be delivered via this method.

In a further embodiment of the invention, the expression vector may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated polynucleotide delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of a polynucleotide *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacteriophage promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacteriophage polymerase.

Another mechanism for transferring expression vectors into cells is receptor-mediated delivery. This approach takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993). Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1993). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been

used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that an adenoviral expression vector also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems, with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of K-*ras* construct in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a polynucleotide into the cells, *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson *et al.*, U.S. Patent 5,399,346, and incorporated herein in its entirety, disclose *ex vivo* therapeutic methods. During *ex vivo* culture, the expression vector can express the antisense K-*ras* construct. Finally, the cells may be reintroduced into the original animal, or administered into a distinct animal, in a pharmaceutically acceptable form by any of the means described below.

D. K-*ras* EXPRESSION VECTORS IN COMBINATION WITH OTHER THERAPIES

Tumor cell resistance to DNA damaging agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992). In the context of the present invention, it is contemplated that antisense K-*ras* therapy could be used similarly in conjunction with chemo- or radiotherapeutic intervention.

To kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with an expression vector and at least one DNA damaging agent. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression vector and the DNA damaging agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the K-*ras* expression vector and the other includes the DNA damaging agent.

Alternatively, the K-*ras* treatment may precede or follow the DNA damaging agent treatment by intervals ranging from minutes to weeks. In embodiments where the DNA damaging factor and K-*ras* expression vector are

applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging agent and expression vector would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either the K-*ras* construct or the DNA damaging agent will be desired. Various combinations may be employed, where K-*ras* is "A" and the DNA damaging agent is "B":

A/B/A	B/A/B	B/B/A	A/A/B	B/B/B/A	B/B/A/B
A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B
A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B

To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

DNA damaging agents or factors are defined herein as any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as

"chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, *e.g.*, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of cisplatin in combination with a K-*ras* antisense expression vector is particularly preferred.

In treating cancer according to the invention, one would contact the tumor cells with a DNA damaging agent in addition to the expression vector. This may be achieved by irradiating the localized tumor site with DNA damaging radiation such as X-rays, UV-light, γ-rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a K-*ras* expression vector, as described above.

Agents that directly cross-link polynucleotides, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been

widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-50 mg/m^2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of polynucleotide precursors and subunits also lead to DNA damage. As such a number of polynucleotide precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves

and UV-irradiation. It is most likely that all of these factors effect a broad range of DNA damage, or the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of K-*ras* expression vectors to patients with K-*ras*-linked cancers will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subject's body. Alternatively, systemic delivery of the expression vector or the DNA damaging agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

Cytokine therapy also has proven to be an effective partner for combined therapeutic regimens. Various cytokines may be employed in such combined approaches. Examples of cytokines include IL-1α IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, G-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, IFN-γ. Cytokines are administered according to standard regimens, as described below, consistent with clinical indications such as the condition of the patient and relative toxicity of the cytokine.

In addition to combining anti-K-*ras*-targeted therapies with chemo-, radio- and cytokine therapies, it also is contemplated that combination with other gene therapies will be advantageous. For example, targeting of K-*ras* and p53 mutations at the same time may produce an improved anti-cancer treatment. Any other tumor-related gene conceivably can be targeted in this manner, for example, p53, p21, Rb, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, other *ras* molecules, *myc, neu, raf, erb, src, fms, jun, trk, ret, gsp, hst, bcl* and *abl*. It also may be desirable to combine anti-sense K-*ras* therapy with an antibody-based gene therapy treatment involving the use of a single-chain antibody construct in which the antibody binds to any of the foregoing molecules.

E. PHARMACEUTICAL COMPOSITIONS AND ROUTES OF ADMINISTRATION

Where clinical application of an adenoviral expression according to the present invention is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the

intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the expression vector, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the

various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation or (ii) elimination of tumor cells. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

In certain embodiments, it may be desireable to provide a continuous supply of therapeutic compositions to the patient. For intravenous or intraarterial routes, this is accomplished by drip system. For topical applications, repeated application would be employed. For various approaches, delayed release formulations could be used that provided limited but constant amounts

of the therapeutic agent over and extended period of time. For internal application, continuous perfusion of the region of interest may be preferred. This could be accomplished by catheterization, post-operatively in some cases, followed by continuous administration of the therapeutic agent. The time period for perfusion would be selected by the clinician for the particular patient and situation, but times could range from about 1-2 h, to 2-6 h, to about 6-10 h, to about 10-24 h, to about 1-2 days, to about 1-2 weeks or longer.

F. KITS

All the essential materials and reagents required for inhibiting tumor cell proliferation may be assembled together in a kit. This generally will comprise selected adenoviral expression vectors. Also included may be various media for replication of the expression vectors and host cells for such replication. Such kits will comprise distinct containers for each individual reagent.

When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred. For *in vivo* use, the expression vector may be formulated into a pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components

are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalent, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

G. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 - **Materials and Methods**

Cell Lines and Culture Conditions. 293 cells (293S, human embryonic kidney cells) at passage thirty-one, grown in minimal essential medium with nonessential amino acids and 10% horse serum, were used for cotransfections. A selected population of 293 cells with faster growing properties (293F) was grown in DMEM[4] with 10% FBS and used for virus amplification. The human NSCLC cell line H460a was maintained in RPMI medium with 5% fetal bovine serum (FBS). This cell line was derived from a large-cell undifferentiated NSCLC line and contains a homozygous mutation at codon 61 of K-*ras* (Mukhopadhyay *et al.*, 1991). To infect 293 and H460a cells with recombinant adenovirus, subconfluent cell monolayers were first incubated with the virus in a minimal amount of complete medium (1 ml/60-mm plate, 37°C in CO_2 incubator, 1 h rocking plates every 10 min to avoid drying). Complete medium was then added and the plates were incubated for 16 h or, in the case of 293 cells, until the appearance of cytopathic effect.

Generation of Recombinant Adenoviruses. An *Nde* I-*Sal* I genomic fragment from the K-*ras* protooncogene, containing exons 2 (176 bp) and 3 (130 bp) with flanking intron sequences and complete intron 2 (1.7 kB) was obtained from the plasmid Apr1-*neo*-Kr*as* (Mukhopadhyay *et al.*, 1991). After blunting the ends with the Klenow, the fragment was cloned between the CMV promoter and SV40 poly A signal in both sense (S) and antisense (AS) orientations. These two expression cassettes and an empty one (to generate another adenoviral vector used as a control) were excised by complete *Cla* I digestion and partial *Xba* I digestion (there is an *Xba* I site in the K-*ras* fragment) and inserted into the polylinker of plasmid pXCJL.1 (F. Graham, McMaster University,

Hamilton, Ontario, Canada) using *Xba* I and *Cla* I. The resulting adenoviral shuttle vectors were cotransfected with pJM17 in 293 cells by DOTAP lipofection (Zhang *et al.*, 1993) to generate AdK*ras*S (sense), AdK*ras*AS (antisense) and AdCMV-pA (empty) vectors. Viruses were subsequently plaque-isolated on 293S cells and amplified in 293F by standard procedures (Zhang *et al.*, 1994; Graham and Prevec, 1991). The viruses were purified by two CsCl gradients (a step gradient of 1.5-1.35-1.25 g/ml, 150,000g 1 h and a continuous gradient of 1.35 g/ml, 150,000g 16 h). After dialysis, stocks were kept at -80°C in a solution containing 10 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$; and 10% glycerol. Titers of purified viruses were determined by plaque assays (Graham and Prevec, 1991).

Northern Blot. Total cellular RNA was isolated from cells with Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of RNA were size-fractionated in MOPS/formaldehyde gels and vacuum transferred to Zeta-Probe GT blotting membranes (BioRad, Hercules, CA). Hybridization and washing were performed according to the manufacturer's instructions. The 2 kB genomic fragment from K-*ras*, labeled by random primer (Redi-prime; Amerisham, Piscataway, NJ) at 2x106 cpm/ml, was used as a probe.

Western Blot. Lysis of infected cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions, size fractionation, transfer, and immunodetection were performed as described elsewhere (Zhang *et al.*, 1994). The antibodies used were: F234-4.2 anti-c-K-*ras* monoclonal antibody (Oncogene Science, Nanhasset, NY) diluted at 1/15; anti-actin monoclonal antibody (Amersham) diluted at 1/3000; and an anti-mouse immunoglobulin horseradish peroxidase-linked

whole antibody from sheep (Amersham, San Diego, CA) diluted at 1/3000.

Proliferation and Colony Formation Assays. For [^{3}H]thymidine uptake assays, cells grown at 50-60% confluence in 60-mm plates were infected for 24 h, trypsinized, counted and seeded in triplicate 96-well plates at 1×10^3 cells/well. At the specified day, 10 μl of a 1:10 dilution of [^{3}H]thymidine (5 Ci/mmol, Amersham) in DMEM with 3% FBS was added to each well and incubated for 4 h. Then cells were washed and harvested to filters for radioactivity counting. Direct cell number assays were performed as described elsewhere (Zhang *et al.*, 1994).

For soft agarose colony formation assays, infected cells were trypsinized, mixed with 0.35% agarose and plated over a base layer of 0.7% agarose as described elsewhere (Zhang *et al.*, 1993). Colonies were counted 10 days later.

Example 2 - Generation of AdKras and AdKrasAS

To construct a recombinant adenovirus expressing a mutated K-*ras*, a 2 kB fragment was inserted downstream of a strong promoter. This fragment was chosen because it has been shown to block p21 protein expression in other systems without affecting the expression of the other proteins of the *ras* family (Zhang *et al.*, 1993). The steps used to construct the virus are parallel to those used to generate the adenoviral vector Ad5CMV-*p53* (Zhang *et al.*, 1994). First, the fragment is inserted in an expression cassette. Second, this cassette is inserted into the E1-deleted region of the Ad5 left arm. And third, this construct is cotransfected with a nonpackageable Ad genome (pJM17). FIG. 1 shows these

steps schematically. The structure of the virus so produced was confirmed by restriction analysis. The *Xba* I sites at the end of exon 3 in the K-*ras* fragment and in front of the CMV promoter allows clear distinction between the sense and the antisense constructs.

Example 3 - **Expression of Antisense K-*ras* RNA in Infected Cells**

The first step in assessing the effect of Ad*KrasAS* is to define an appropriate range of dose and toxicity. It was assumed that the more antisense RNA present in the cell, the stronger the growth inhibitory-effects would be, with a limit imposed by the toxic effects of large doses of viral proteins. To determine the optimal therapeutic ratio, H460a cells were infected with an adenovirus expressing the β-gal gene (Ad5CMV-*LacZ*; Zhang *et al.*, 1994), at an increasing multiplicity of infection (MOI). At an MOI of 100 pfu/cell, 65% of cells were transduced with the β-gal gene, as determined by X-gal staining. To reach a complete transduction of H460a cells, an MOI of about 1000 pfu/cell was required. In preliminary tests of toxicity in H460a cells using several control viruses such as Ad5CMV-*LacZ*, Ad*KrasS* and AdCMV-pA, it was found that MOI's higher than 400 pfu/cell reduced cell proliferation. Taking these observations into account, an MOI of 100 pfu/cell was chosen to test the effect of Ad*KrasAS*.

To determine the effects of Ad*KrasAS* at the mRNA level, cells were infected with Ad*KrasS* or with Ad*KrasAS*, and total RNA extracted after 1, 3 and 5 days was analyzed by Northern blot. Cells infected with the control virus Ad*KrasS* expressed the same K-*ras* RNA levels as noninfected cells. The size of the transcript that arose from Ad*KrasS* appeared to be approximately 4 kB,

which indicates a possible read-through of the SV40 polyA signal. In cells infected with the antisense AdK*ras*AS virus, the endogenous K-*ras* RNA transcript detected in noninfected cells was no longer detected, and other transcripts of approximately 4 and 6 kB appeared, presumable arising from AdK*ras*AS. These results, reproducibly confirmed, indicate that AdK*ras*AS affects the pattern of K-*ras* mRNA expression.

Protein production was analyzed by Western blot using a monoclonal antibody specific for the p21 protein. Three days after infection with AdK*ras*AS at an MOI of 100 pfu/cell (65% of cells transduced), the level of p21 protein was less than half (30%) of that found in uninfected cells or in cells infected with AdK*ras*S or AdCMV-*LacZ*. As an internal control, an anti-actin-specific antibody was added in the incubation step. Actin levels were the same irrespective of the treatment, indicating that the observed reduction in p21 protein was specific to the antisense virus.

Example 4 - **Growth Inhibition by AdK*ras*AS**

If translation of the p21 protein was blocked by the antisense RNA from AdK*ras*AS, it would be expected that the virus would have a growth-inhibitory effect. To examine this, the growth of a population of cells infected with AdK*ras*AS was compared to that of uninfected cells, or infected with control virus. In wells to which AdK*ras*AS was added, cell number and [^{3}H]thymidine incorporation in a 4 h period were consistently (three independent studies) about 50% of those in controls (FIG. 2). After 4 days, although the cell number was lower, the proliferation rate of the remaining cells was the same as the uninfected or control-infected populations, suggesting that these cells arose from non-transduced

cells. No cells were observed for more than 5 days, because in control treatments, the monolayer was reaching confluence and cell number and thymidine incorporation began to plateau after this period. When an MOI higher than 400 pfu/cell was used in an attempt to transduce all cells, cell number and thymidine incorporation proportionally decreased in wells with control viruses. The observed magnitudes of nonspecific toxicity were: Ad5CMV-*LacZ* > AdCMV-pA > AdK*ras*S.

Another approach used to study the growth-inhibitory effect of AdK*ras*AS was to test the colony-forming ability of transduced cells. Plates with H460a cells infected with AdK*ras*AS consistently (three studies) showed about ten-fold fewer colonies; most cells remained as single cells (number of colonies, 121±24), as compared with uninfected cells (1304±182), AdK*ras*S-infected cells (1275±165) and Ad5CMV-*LacZ*-infected cells (118±134). Thus, AdK*ras*AS markedly decreased the capacity of human lung cancer cells to achieve anchorage-independent growth.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM
(B) STREET: 201 WEST 7TH STREET
(C) CITY: AUSTIN
(D) STATE: TEXAS
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 78701

(ii) TITLE OF INVENTION: ADENOVIRUS-ANTISENSE K-RAS EXPRESSION VECTORS AND THEIR APPLICATION IN CANCER THERAPY

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/550,959
(B) FILING DATE: 31-OCT-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3220 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTGCGAACAT TGGTGTACAT GTATCTGTTT GAGTCCCTGT TTAGTTATTT TGGTTATATA 180

CCTAGGAATG GAATGCTGAT CATATGGTAA TTCTGTGTTT AACTTTTGAG GAACTACCAC 240

TGTTTTCCAC AATGGCATCA CCCATTTTAC ATTCCCACCA GCAATGCACA AAGATTTCAG 300

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GGAGTAACTT GCTCCAGACT GCATCGGTAG TGGTGGTGCT GGGATTGAAA CCTAGGCCTG 1020

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 290 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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AGGACCATTC TTTGATACAG ATAAAGGTTT CTCTGACCAT TTTCATGAGT 290

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 789 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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ACACTTATTT TCCATTTTTT GAGTTTTTTT GTTTTGTTTT TTTAATAATA GCCAATCCTA 120

ATGGGTATGT GGTAGCATCT CATGGTTTTG ATTTTATTTT CCTGACTATT GATGATGTTG 180

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 615 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 866 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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CAAGGTGGCA ACCACTTTAG AACTACTTAG GTGTAGTATT CTAACTTGAA GTATTAAAAG 840

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 617 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAATTCTAAA AGTCCTAATA TATGTAATAT ATATTCAGTT GCCTGAAGAG AAACATAAAG 60

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CATTAGTTCG AGAAATTCGA AAACATAAAG AAAAGATGAG CAAAGATGGT AAAAAGAAGA 240

AAAAGAAGTC AAAGACAAAG TGTGTAATTA TGTAAATACA ATTTGTACTT TTTTCTTAAG 300

GCATACTAGT ACAAGTGGTA ATTTTTGTAC ATTACACTAA ATTATTAGCA TTTGTTTTAG 360

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TTTGGTGCAT GCAGTTGATT ACTTCTTATT TTTCTTACCA AGTGTGAATG TTGGTGTGAA 600

ACAAATTAAT GAAGCTT 617

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 574 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..567

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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 1               5                  10                  15

AGT GCC TTG ACG ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GAA TAT      96
Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr
            20                  25                  30
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GAT CCA ACA ATA GAG GAT TCC TAC AGG AAG CAA GTA GTA ATT GAT GGA      144
Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly
          35                  40                  45

GAA ACC TGT CTC TTG GAT ATT CTC GAC ACA GCA GGT CAA GAG GAG TAC      192
Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
     50                  55                  60

AGT GCA ATG AGG GAC CAG TAC ATG AGG ACT GGG GAG GGC TTT CTT TGT      240
Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
 65                  70                  75                  80

GTA TTT GCC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT      288
Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
                 85                  90                  95

AGA GAA CAA ATT AAA AGA GTT AAG GAC TCT GAA GAT GTA CCT ATG GTC      336
Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Glu Asp Val Pro Met Val
            100                 105                 110

CTA GTA GGA AAT AAA TGT GAT TTG CCT TCT AGA ACA GTA GAC ACA AAA      384
Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr Val Asp Thr Lys
        115                 120                 125
```

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CAG GCT CAG GAC TTA GCA AGA AGT TAT GGA ATT CCT TTT ATT GAA ACA      432
Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Glu Thr
    130                 135                 140

TCA GCA AAG ACA AGA CAG AGA GTG GAG GAT GCT TTT TAT ACA TTG GTG      480
Ser Ala Lys Thr Arg Gln Arg Val Glu Asp Ala Phe Tyr Thr Leu Val
145                 150                 155                 160

AGA GAG ATC CGA CAA TAC AGA TTG AAA AAA ATC AGC AAA GAA GAA AAG      528
Arg Glu Ile Arg Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys
                165                 170                 175

ACT CCT GGC TGT GTG AAA ATT AAA AAA TGC ATT ATA ATG TAATCTG          574
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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 189 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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1 5 10 15

Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr
20 25 30

Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly
35 40 45

Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
50 55 60

Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
65 70 75 80

Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
85 90 95

Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Glu Asp Val Pro Met Val
100 105 110

Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr Val Asp Thr Lys
115 120 125

Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Glu Thr
130 135 140

Ser Ala Lys Thr Arg Gln Arg Val Glu Asp Ala Phe Tyr Thr Leu Val
145 150 155 160

Arg Glu Ile Arg Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys
165 170 175

Thr Pro Gly Cys Val Lys Ile Lys Lys Cys Ile Ile Met
180 185

6. **CLAIMS:**

1. An adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-*ras* polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-*ras* transcript synthesized therefrom is antisense.

2. The adenoviral expression vector according to claim 1, further comprising a polyadenylation signal.

3. The adenoviral expression vector according to claim 1, further comprising a selectable marker.

4. The adenoviral expression vector according to claim 1, wherein said polynucleotide consists essentially of exon 2, intron 3 and exon 3.

5. The adenoviral expression vector according to claim 1, wherein said polynucleotide consists essentially of the 1.7 kB *Nde* I-*Sal* I fragment of the K-*ras* protooncogene.

6. The adenoviral expression vector according to claim 1, wherein said expression vector is replication deficient.

7. The adenoviral expression vector according to claim 6, wherein said expression vector lacks at least a portion of the E1 region.

8. A pharmaceutical composition comprising (i) an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-*ras* polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-*ras* transcript synthesized therefrom is antisense and (ii) a pharmaceutically acceptable buffer, solvent or diluent.

9. A method for inhibiting K-*ras* function in a cell comprising the steps of:

(i) providing an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-*ras* polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-*ras* transcript synthesized therefrom is antisense; and

(ii) contacting said expression vector with said cell.

10. The method according to claim 9, wherein said cell is a transformed cell and said contacting reverses said transformed phenotype.

11. The method according to claim 10, wherein said cell is a tumor cell.

12. The method according to claim 11, wherein said tumor cell is a lung cancer, pancreatic cancer or colon cancer cell.

13. The method according to claim 9, wherein said expression vector is packaged in an adenoviral capsid and said contacting comprises infecting said cell.

14. A method of treating a mammal with cancer comprising:

(i) providing a pharmaceutical composition comprising (a) an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-*ras* polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-*ras* transcript synthesized therefrom is antisense, and (b) a pharmaceutically acceptable buffer, solvent or diluent; and

(ii) administering said pharmaceutical composition to said mammal.

15. The method according to claim 14, wherein said mammal is a human.

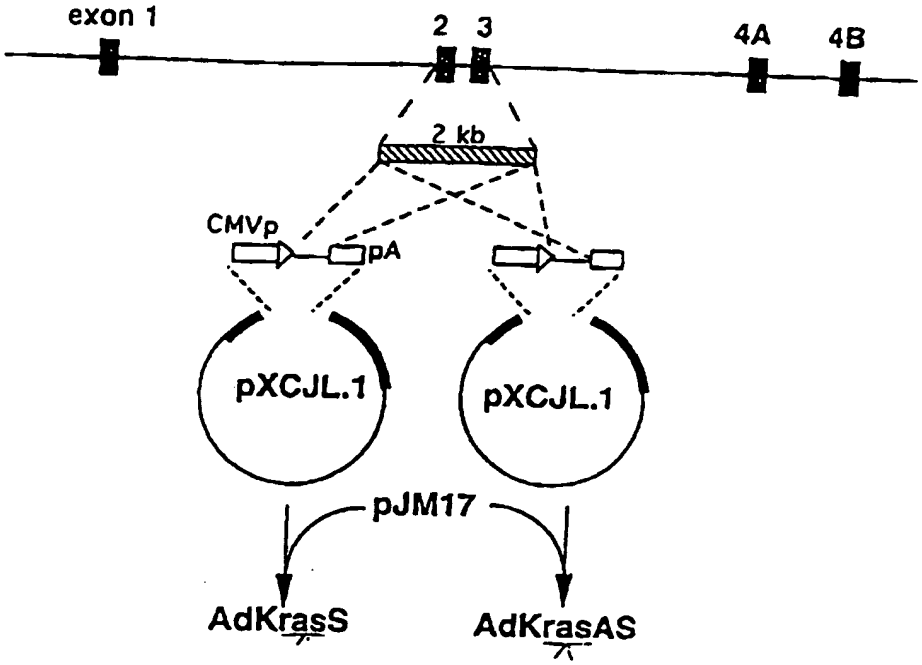
16. The method according to claim 15, wherein said administering is via intravenous injection.

17. The method according to claim 15, wherein said administering is via orthotopic injection.

18. The method according to claim 14, wherein said cancer is lung cancer, pancreatic cancer or colon cancer.

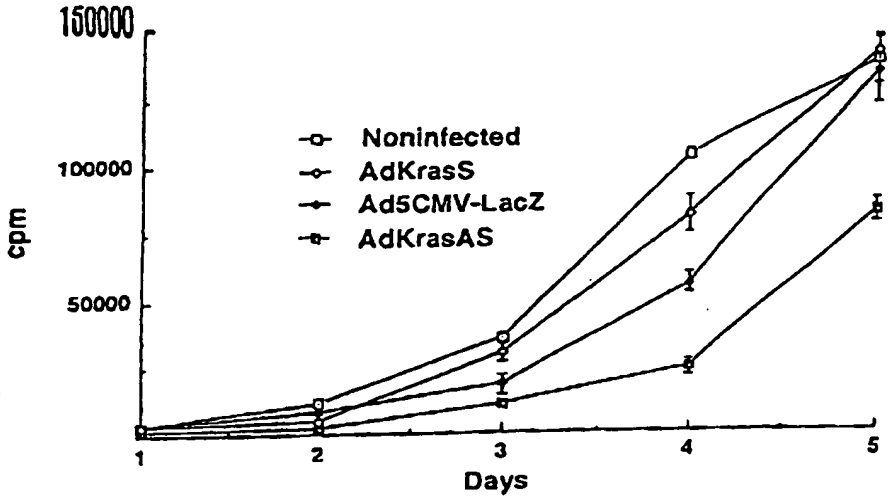
19. The method according to claim 14, wherein said expression vector is packaged in an adenoviral capsid and said contacting comprises infecting said cell.

20. A kit comprising, in suitable container means, an expression vector comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a K-*ras* antisense transcript, wherein said nucleic acid is under transcriptional control of said promoter, and a pharmaceutically acceptable buffer, solvent or diluent.



ADENOVIRAL VECTOR CONSTRUCTION

FIG. 1



GROWTH CURVE OF TRANSDUCED H460a CELL IN VITRO

FIG. 2

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/17979

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/86 A61K31/70 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 15680 A (UNIV TEXAS) 17 September 1992 see page 6, line 17 - page 10, line 25 see page 21, line 1 - line 12	1-5, 9-12,14, 15,18,20
Y	see claims	1,6-8, 13,16, 17,19

Y	WO 95 02697 A (RHONE POULENC RORER SA ;PERRICAUDET MICHEL (FR); VIGNE EMMANUELLE) 26 January 1995 see page 3, line 9 - line 25 see page 6, line 25 - line 30 see examples see claims 1,12,14,28-30	1,6-8, 13,16,19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1997	04.03.97

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/17979

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER RES., (1993) 53/8 (1743-1746), XP002025920 GEORGES R.N. ET AL: "Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K - ras construct." cited in the application see the whole document	17
O,A	PROC ANNU MEET AM ASSOC CANCER RES, (MARCH 1994). VOL. 35, PP. 692-3., XP002025921 ROTH. J. A.: "Gene replacement strategies for therapy and prevention of lung cancer."	
O,A	ANTISENSE RES.DEV. 5 (SPRING 95); 109; OP-33 , XP002025922 FUNATO, T. ET AL.: "Effects of tumor growth suppression in cancers by antisense-ras ribozyme" & 1ST INT.ANTISENSE CONF.JAPAN (04 TO 07-12-94),	
A	HUMAN GENE THERAPY, vol. 4, 1 January 1993, pages 461-476, XP002004336 RICH, D. ET AL.: "DEVELOPMENT AND ANALYSIS OF RECOMBINANT ADENOVIRUSES FOR GENE THERAPY OF CYSTIC FIBROSIS" cited in the application	
P,X	CANCER GENE THERAPY, (1996 SEP-OCT) 3 (5) 296-301., XP000645028 ALEMANY, R. ET AL.: "Growth inhibitory effect of anti- K - ras adenovirus on lung cancer cells." see the whole document	1-7, 9-14,20

1

INTERNATIONAL SEARCH REPORT

.....rnational application No.
PCT/US 96/ 17979

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 9-13
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 9-13 (as far as in vivo methods are concerned) and 14-19 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter nal Application No
PCT/US 96/17979

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9215680 A	17-09-92	AU 663702 B	19-10-95
		AU 1570492 A	06-10-92
		CA 2108144 A	07-09-92
		EP 0575518 A	29-12-93
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		FR 2718749 A	20-10-95
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		JP 8501703 T	27-02-96
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		NZ 269156 A	26-03-96
		PL 308122 A	24-07-95
		SK 31295 A	08-05-96
		ZA 9405012 A	20-02-95